Phosphorylation of the Nicotinic Acetylcholine Receptor by Protein Tyrosine Kinases[a]

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Protein phosphorylation is a major posttranslational modification widely recognized to regulate almost all cellular processes.[1,2] Based on their amino acid specificities, protein kinases may be classified as either protein serine/threonine kinases or protein tyrosine kinases. Protein tyrosine kinases were originally identified as retroviral oncogene products and subsequently shown to have normal cellular homologs.[3] Initially, protein tyrosine kinases were believed to be important primarily in the regulation of growth and mitogenesis. However, more recent studies implicate protein tyrosine kinases in the regulation of differentiated cell function. For example, many protein tyrosine kinases are most highly expressed in the neurons of the central nervous system.[4] Furthermore, protein tyrosine kinases can mediate neuronal differentiation; activation of the nerve growth factor receptor[5] or expression of v-*src*[6] in PC12 chromaffin cells induces a sympathetic neuron-like phenotype. These studies suggest an involvement of protein tyrosine kinases in neuronal differentiation as well as in synaptic transmission.

The transfer of information from a neuron to its target is the process of synaptic transmission. Chemical synapses, at which the postsynaptic response is mediated by ligand-gated ion channels, provide many opportunities for synaptic plasticity. Our understanding of the molecular mechanisms underlying synapse formation, function, and modulation derives primarily from studies examining the neuromuscular junction. At the neuromuscular junction, the acetylcholine receptor (AChR) is the ligand-gated ion channel that mediates the rapid excitatory postsynaptic response of the muscle. Because of its enrichment in the electric organs of *Torpedo californica,* the AChR has served as a model for the study of the structure, function, and regulation of ligand-gated ion channels. The AChR is a 250-kDa pentameric complex comprised of four homologous subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$ (FIG. 1 and ref. 7). At least three protein kinase activities phosphorylate the AChR (FIG. 1 and ref. 8). Protein kinase A phosphorylates the γ and δ subunits, protein kinase C phosphorylates the δ subunit, and a protein tyrosine kinase activity phosphorylates the β, γ, and δ subunits. Phosphorylation of the AChR by all three protein kinase

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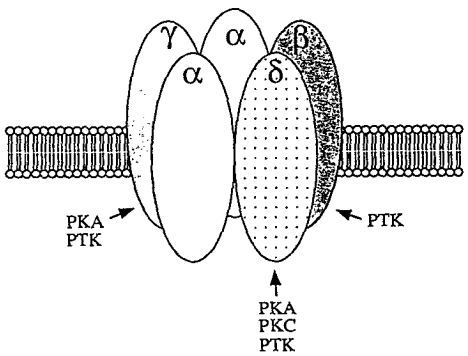

FIGURE 1. Model of the structure and subunit specificity for phosphorylation of the AChR. The α, β, γ, and δ subunits are as indicated. The specificity for phosphorylation of the AChR subunits by protein kinase A (PKA), protein kinase C (PKC), and protein tyrosine kinase(s) (PTK) are as indicated.

activities results in an alteration of channel desensitization kinetics.[8] In addition, activation of protein tyrosine kinase activity, including tyrosine phosphorylation of the AChR, is associated with a clustering of the AChR during the formation of postsynaptic specializations. In this report, we review some of our recent studies to identify the extracellular factors that stimulate AChR tyrosine phosphorylation as well as the protein tyrosine kinase(s) that may be involved in the regulation of synaptic function at the neuromuscular junction by directly phosphorylating the AChR.

Methodologies used are as described in references 9–12.

RESULTS

An Extracellular Factor That Stimulates AChR Tyrosine Phosphorylation

The AChR of *Torpedo californica* electric organ and innervated rat skeletal muscle is phosphorylated on tyrosine residues.[9,13,14] In contrast, the AChR of cultured myotubes[15] and BC3H1 myocytes[16] contains very little phosphotyrosine. These studies suggest that innervation may regulate the tyrosine phosphorylation of the AChR. Another effect of the neuron on the AChR is to induce receptor clustering at the site of nerve and muscle contact.[17] In addition, some reports have suggested that phosphorylation of the AChR may be involved in regulating receptor distribution.[10,18-20] It is now known that the neuronally derived extracellular maxtrix protein agrin mediates AChR clustering induced by the neuron.[21] Therefore, we have examined whether the effect of agrin to stimulate AChR aggregation is associated with phosphorylation of the receptor by a protein tyrosine kinase.

Cultured chick myotubes were labeled with anti-phosphotyrosine antibodies and rhodamine-conjugated α-bungarotoxin. The small number of aggregates of AChRs found on the surface of control myotubes show only slight labeling with the anti-phosphotyrosine antibody (FIG. 2A and B). After 4 h of agrin treatment, AChR aggregates increase in size and the clusters stain intensely for phosphotyrosine (FIG. 2C and D). There is a striking correspondence in the localization of the expanding AChR clusters and phosphotyrosine during the time course of agrin treatment (FIG. 2C–H). Thus, tyrosine-phosphorylated proteins aggregate at agrin-induced AChR clusters.

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To determine whether the AChR itself is phosphorylated in response to agrin, chick myotube cultures were treated with or without agrin and, after affinity purification, the AChR phosphotyrosine content was examined by Western blotting using the α-phosphotyrosine antibody. Agrin induces a dramatic increase in the phosphorylation of the chicken AChR β subunit on tyrosine residue(s) (FIG. 3). Thus, agrin induces both AChR clustering and tyrosine phosphorylation.

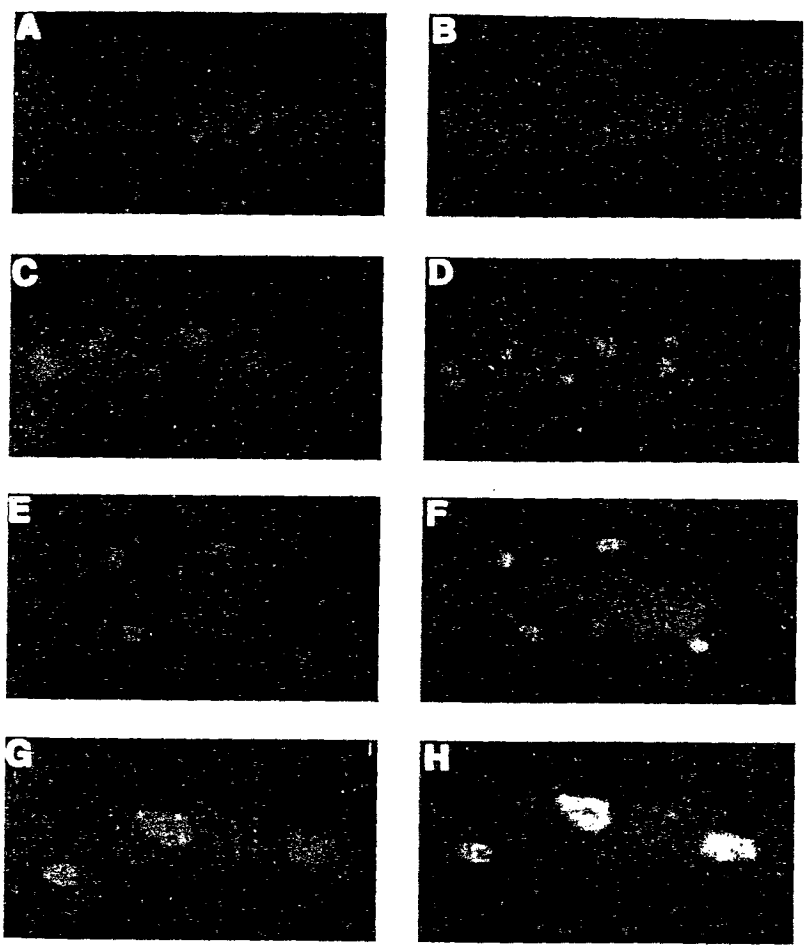

FIGURE 2. Effect of agrin on AChR and phosphotyrosine aggregation. Fluorescence micrographs of segments of cultured chick myotubes treated with agrin for 0 h (**A** and **B**), 4 h (**C** and **D**), 8 h (**E** and **F**), and 18 h (**G** and **H**) were fixed, permeabilized, and labeled with rhodamine-conjugated α-bungarotoxin to reveal the distribution of the AChR (**A, C, E,** and **G**) and with antiphosphotyrosine monoclonal antibody PY20 and a fluorescein-conjugated secondary antibody to visualize phosphotyrosine residues (**B, D, F,** and **H**) as described.[10] (From Wallace *et al.*[10] Reproduced, with permission, from *Neuron.*)

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Identification of Synaptic Protein Tyrosine Kinases That Associate with the AChR

Because of the enrichment of synaptic components, including the AChR, in *Torpedo californica* electric organ, we predicted that the kinase that phosphorylates the AChR would also be enriched in that tissue. Therefore, our initial goal was to identify protein tyrosine kinases of *Torpedo californica* electric organ by molecular cloning. Protein tyrosine kinases contain several highly conserved subdomains within the catalytic region[22] making them particularly amenable to analysis by the polymerase chain reaction (PCR).[23] Using cDNA derived from *Torpedo* electric organ as template, we used oligonucleotides based on the sequences of subdomains VII and VIII of the *src* class of protein tyrosine kinases to generate PCR products encoding protein tyrosine kinase fragments. *Src*-like protein tyrosine kinase cDNA clones were identified by screening a *Torpedo* electric organ γgt10 cDNA library with the

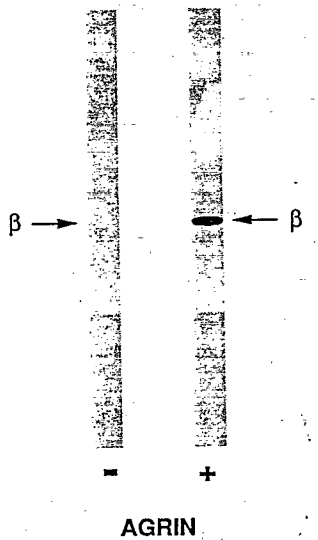

FIGURE 3. Effect of agrin on the phosphotyrosine content of the AChR. Cultured chicken myotubes were treated without (−) or with (+) agrin for 12 h after which the AChR was isolated, resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed for phosphotyrosine content using a monoclonal antibody as described.[10] The position of the β subunit is as indicated. (Modified from Wallace *et al.*[10] and used with permission from *Neuron.*)

cloned PCR products. Positive clones could be divided into two classes based on Southern analysis using the PCR products.

Within the coding regions, the DNA sequences of the two clones have 76% dispersed identity (with three gaps) demonstrating that they are derived from homologous but unique genes (data not shown). Each deduced translation product encodes an Src-like protein tyrosine kinase that contains all the hallmark subdomains including a myristylation site, Src homology 3 (SH3) and Src homology 2 (SH2) domains, and all the recognized subdomains of the catalytic region (FIG. 4). The amino acid sequences of the two clones show 82% identity to each other. Homology searches demonstrated that both clones are most similar to *fyn,* a member of the *src* class of protein tyrosine kinases (FIG. 4). Two *fyn* protein tyrosine kinases, the neuronal and thymic variants, have been identified; these forms arise from alternative splicing.[24,25,28] As shown in FIGURE 4, both *Torpedo* clones contain the neuronal alternative splice insert, suggesting one of the clones is the *Torpedo* neuronal *fyn* and

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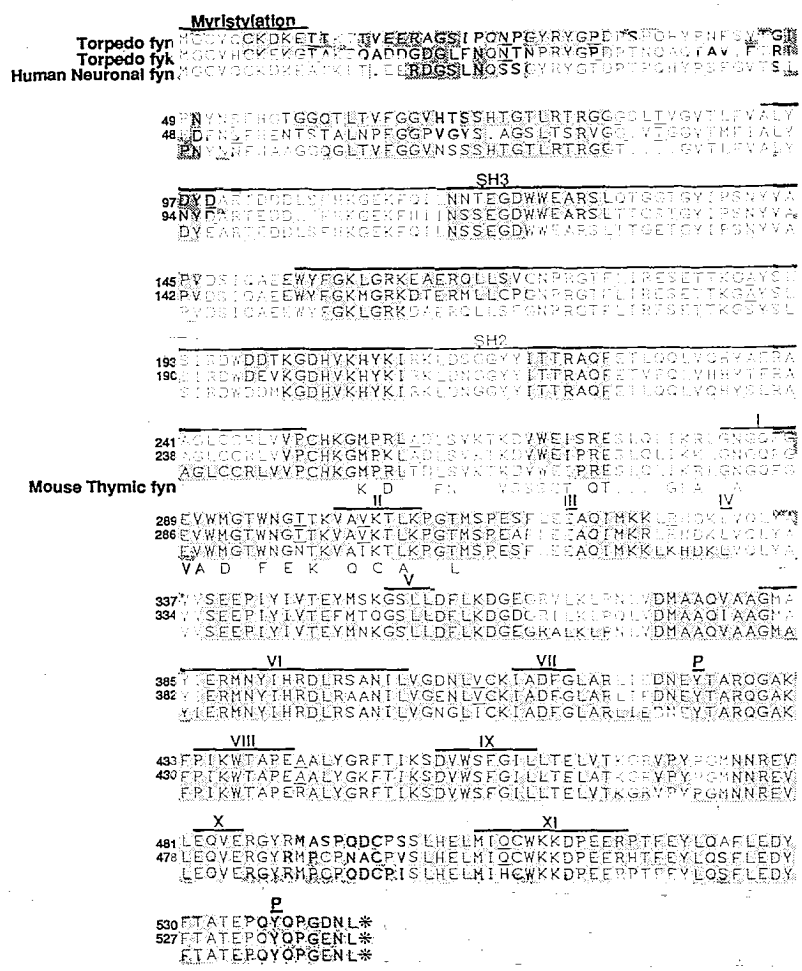

FIGURE 4. Complete amino acid sequences of two protein tyrosine kinases expressed in *Torpedo* electric organ. The homologies of the deduced amino acid sequences of the two *Torpedo* electric organ protein tyrosine kinases to known proteins were determined as described.[11] For each clone, amino acid identities with the neuronal form of human Fyn[24] are indicated by shading. Amino acids of nonidentity with human Fyn but identity between Fyn and Fyk are indicated by underlining. The divergence between the neuronal and the alternatively spliced thymic form of murine Fyn[25] is shown. Conserved protein tyrosine kinase subdomains are indicated by horizontal bars. SH3 and SH2, Src homology domains 3 and 2;[26,27] P, putative phosphorylation sites; I–XI, conserved subdomains.[22] The amino acid positions in the sequence of each clone are indicated on the left. (From Swope and Huganir.[11] Reproduced, with permission, from the *Journal of Biological Chemistry.*)

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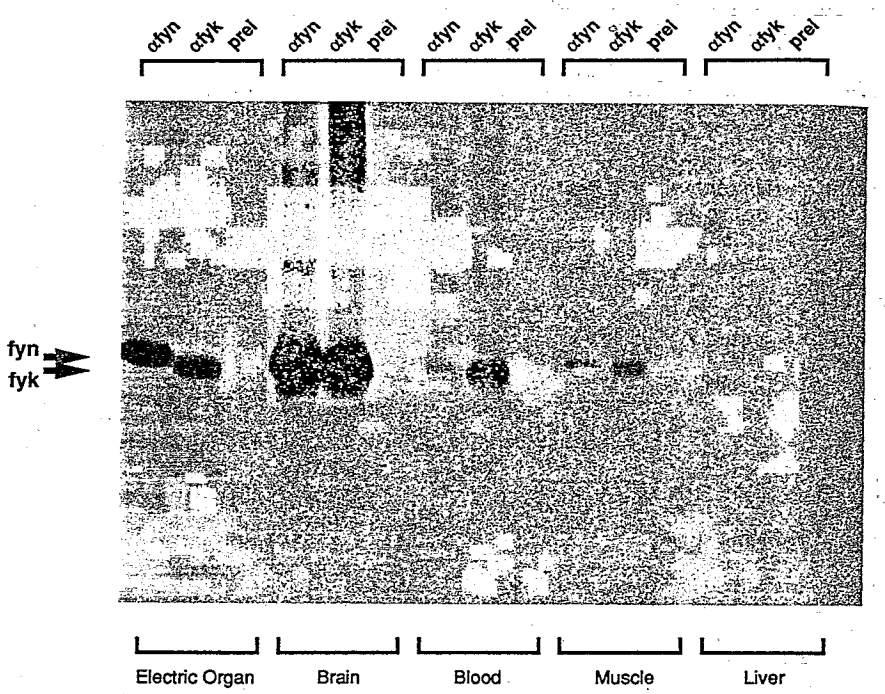

FIGURE 5. Expression of Fyn and Fyk protein tyrosine kinase activities in various *Torpedo* tissues. Membrane proteins (500 μg) prepared from the tissues indicated were solubilized at 1 mg/mL, immunoprecipitated with anti-*fyn* (αfyn), anti-*fyk* (αfyk), or preimmune (prei) serum as indicated and incubated under phosphorylating conditions at 30 °C for 30 min in the presence of [γ-^{32}P]ATP followed by analysis by SDS-PAGE and autoradiography for 1 h as described.[11] (From Swope and Huganir.[11] Reproduced, with permission, from the *Journal of Biological Chemistry.*)

the other clone a novel gene product. The upper clone in FIGURE 4 shows 78% identity with human neuronal *fyn* at the nucleotide level and 90% identity at the amino acid level. The second clone shows 75% identity with human neuronal *fyn* at the nucleotide level and 82% at the amino acid level. Between members of the *src* family of protein tyrosine kinases, the amino acid sequence between the myristylation motif and the SH3 domain is the region that is most unique.[29] Therefore, to clarify the identity of our two clones, we compared the unique domain of each clone to known protein tyrosine kinases. The upper clone in FIGURE 4 is most homologous to *fyn,* showing 69% identity. In contrast, the second clone has only 39% identity with *fyn* in this N-terminal region. In addition, the second clone is almost equally homologous, 32% identity, with the *yes* protein tyrosine kinase.[30] These data support the upper clone in FIGURE 4 as the *Torpedo* neuronal *fyn* protein tyrosine kinase. The second clone is a novel protein tyrosine kinase that we have named *fyk* for *fyn-yes*-kinase.

To examine the expression of these two tyrosine kinases at the protein level, we generated antibodies to bacterial fusion proteins containing the N-terminal unique

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domains of *fyn* and *fyk*. These antibodies were used in an immunoprecipitation/kinase assay in which the immunoprecipitated proteins were incubated under phosphorylating conditions in the presence of [γ-^{32}P]-ATP. Two protein tyrosine kinase activities of *Torpedo* electric organ are distinguished with antibodies to *fyn* and *fyk* (FIG. 5). The antibody to *fyn* immunoprecipitated a 55-kDa protein that contained only phosphotyrosine (data not shown), presumably due to autophosphorylation. In contrast, anti-*fyk* antibodies immunoprecipitated a doublet of 56- and 53-kDa (FIG. 5). The appearance as a doublet may be due to differing phosphorylation states because the immunoprecipitated phosphorylated Fyk contained both phosphotyrosine and phosphoserine (data not shown). The distributions of Fyn and Fyk protein tyrosine kinase activities in various *Torpedo* tissues were examined using the immunoprecipitation/kinase assay. *Torpedo* electric organ and brain are highly enriched in both kinases (FIG. 5). In addition, Fyn and Fyk activities are detected in muscle, a tissue that also expresses AChR and from which electric organ is embryonically derived. It is interesting to note that the relative abundance of the two kinases in electric organ and muscle is proportional to the expression of the AChR in these tissues.

Protein tyrosine kinases of the Src class are typically associated with the membrane. *Torpedo* electroplax membranes can be fractionated on discontinuous sucrose gradients to enrich for postsynaptic versus nonpostsynaptic membranes.[9,31] We determined the contribution of Fyn and Fyk to the total protein tyrosine kinase activity present in postsynaptic membranes of *Torpedo* electric organ. Detergent extracts of postsynaptic membranes were depleted of Fyn and Fyk by immunoprecipitation after which the kinase activity in the supernatant was measured using the synthetic polypeptide substrate POLY (Glu-Na,Tyr)4:1. Phosphorylation of POLY (Glu-Na,Tyr)4:1 by solubilized membrane proteins after immunoprecipitation with preimmune serum was defined as 100% of the protein tyrosine kinase activity (FIG. 6). When the membrane proteins are immunoprecipitated with the anti-*fyn* antibody, the kinase activity in the supernatant is reduced to 66% of control (FIG. 6). Thus, Fyn

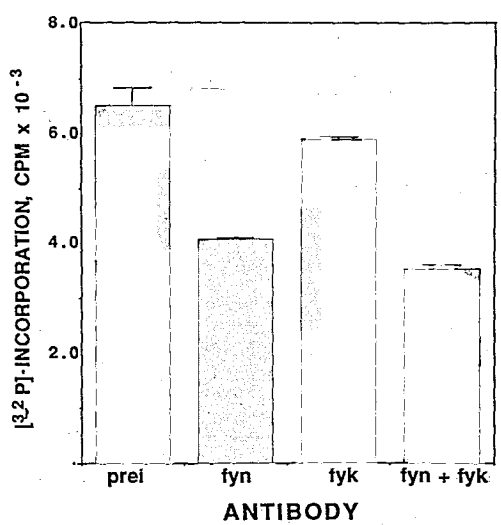

FIGURE 6. Percentage of protein tyrosine kinase activity of postsynaptic membranes represented by Fyn and Fyk. Postsynaptic membranes proteins (25 μg) of *Torpedo* electric organ were solubilized at 1 mg/mL and immunoprecipitated with preimmune (prei), anti-*fyn* (fyn), anti-*fyk* (fyk), or anti-*fyn* plus anti-*fyk* (fyn + fyk) serum as indicated. The supernatants were removed and incubated with poly (Glu-Na,Tyr), 4:1 at 1 μg/μL under phosphorylating conditions in the presence of [γ-^{32}P]ATP. After 30 min at 30 °C the reactions were stopped, and the supernatants were analyzed for ^{32}P incorporation as described.[11] The data represent the mean value ± SE, n = 5. (From Swope and Huganir.[11] Reproduced, with permission, from the *Journal of Biological Chemistry.*)

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represents 34% of the total protein tyrosine kinase activity. In the experiment shown in FIGURE 6, anti-*fyk* antibody depleted 4% of the kinase activity whereas anti-*fyn* plus anti-*fyk* decreased the activity to 57% of control. Thus, Fyn plus Fyk constituted 43% of the kinase activity. In three experiments, Fyn and Fyk represent 36 ± 2% and 8 ± 3%, respectively of the total activity (mean ± SEM). Thus, together these two protein tyrosine kinases comprise a major fraction (44 ± 4%) of the protein tyrosine kinase activity in the postsynaptic membrane.

A "kinase trapping" strategy has been used by others to show that Fyn as well as the Lyn and Yes protein tyrosine kinases are associated with $p21^{ras}$GAP, a tyrosine-phosphorylated substrate.[32] We used the same strategy to examine whether Fyn or Fyk are in a complex with the AChR. To examine whether tyrosine kinase activity is associated with the receptor, the AChR was isolated from detergent extracts of postsynaptic membranes by immunoprecipitation with a monoclonal antibody (88b) and then incubated under phosphorylating conditions. As shown in FIGURE 7A, the β, γ, and δ subunits of the AChR are phosphorylated in the immunoprecipitate. Phosphoamino acid analysis demonstrated that all three subunits contain phosphotyrosine (data not shown). These data suggest that a protein tyrosine kinase is coimmunoprecipitated with the AChR. To examine whether Fyn or Fyk is complexed with the AChR, we did the reciprocal experiment shown in FIGURE 7B. Postsynaptic membranes solubilized under stringent conditions were incubated with either preimmune, anti-*fyn*, or anti-*fyk* antibodies and then the immunoprecipitates were analyzed for the presence of the AChR using a pool of monoclonal antibodies to the individual subunits. Immunoprecipitation with anti-*fyn* and anti-*fyk* antibodies

FIGURE 7. Coimmunoprecipitation of the AChR with Fyn and Fyk. (**A**) Postsynaptic membrane proteins (150 μg) prepared from *Torpedo* electric organ were solubilized at 1 mg/mL, immunoprecipitated in the presence (+88b) or absence (−) of monoclonal antibody 88b to the AChR, and incubated at 30 °C for 60 min in the presence of [γ-^{32}P]ATP with inhibitors of both protein kinase A and protein kinase C followed by analysis by SDS-PAGE and autoradiography for 6 hr as described.[11] The positions of the AChR β, γ, and δ subunits are as indicated. (**B**) Postsynaptic membrane proteins (250 μg) were solubilized at 1 mg/mL with immunoprecipitation buffer containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS and then immunoprecipitated with preimmune (prei), anti-*fyn* (αfyn), or anti-*fyk* (αfyk) serum in the absence or presence of kinase-specific fusion protein (+FP) or backbone fusion protein (+BB). The immunoprecipitates were resolved by SDS-PAGE, blotted onto nitrocellulose, and analyzed with a pool of monoclonal antibodies to the AChR as described.[11] The positions of the AChR α, β, γ, and δ subunits are as indicated. (**C**) Postsynaptic membrane proteins (250 μg) were solubilized at 1 mg/mL with immunoprecipitation buffer containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS and then immunoprecipitated with preimmune (prei), anti-*fyn* (αfyn), or anti-*fyk* (αfyk) serum. The immunoprecipitates and 10 μg of *Torpedo* electric organ postsynaptic membrane proteins (PSM) were resolved by SDS-PAGE, blotted onto nitrocellulose, and analyzed with a monoclonal antibody to the 43-kDa protein as described.[11] The position of 43 kDa is as indicated. (**D**) Partially purified AChR (250 μg), prepared as described,[11] was immunoprecipitated with preimmune (prei), anti-*fyn* (αfyn), or anti-*fyk* (αfyk) antiserum. The immunoprecipitates were incubated at 30 °C for 30 min in the presence of [γ-^{32}P]ATP followed by analysis by SDS-PAGE and autoradiography for 4 h as described.[11] The positions of the 50-kDa (β), 58-kDa (γ), and 65-kDa (δ) proteins are as indicated. (**E**) Partially purified AChR (25 μg), prepared as described,[11] was immunoprecipitated with preimmune (prei), anti-*fyn* (αfyn), or anti-*fyk* (αfyk) serum. The immunoprecipitates were resolved by SDS-PAGE, blotted onto nitrocellulose, and analyzed with a pool of monoclonal antibodies to the AChR as described.[11] The positions of the AChR α, β, γ, and δ subunits are as indicated. (From Swope and Huganir.[11] Reproduced, with permission from the *Journal of Biological Chemistry*.)

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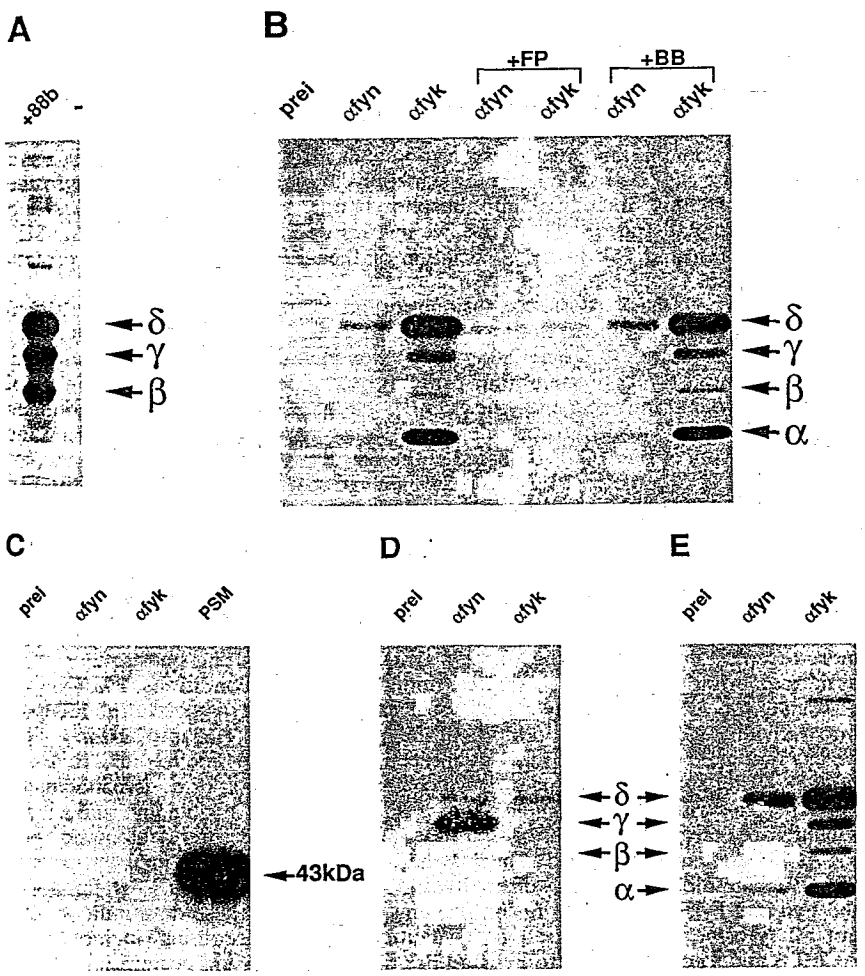
results in specific coimmunoprecipitation of the AChR, with the anti-*fyk* immunoprecipitates being more enriched in AChR than the anti-*fyn* immunoprecipitates. The coimmunoprecipitation of the AChR with Fyn and Fyk is blocked when the antibodies are preincubated with the kinase-specific fusion proteins used as immunogens, but not with the backbone fusion protein (FIG. 7B). These data indicate that the complex formation is due to an association of the AChR with the kinases. In addition, coimmunoprecipitation of the receptor with Fyn and Fyk is apparently not due to a nonspecific association of the abundant AChR with the kinases because the 43-kDa protein, another synaptic component that is stoichiometrically expressed with the receptor,[33] is not coimmunoprecipitated with Fyn and Fyk (FIG. 7C). Taken together, these data indicate that the AChR forms a specific and tight complex with



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Fyn and Fyk, suggesting that these kinases may catalyze the tyrosine phosphorylation of the receptor.

Association of the AChR with Fyn and Fyk is also supported by the experiment shown in FIGURE 7D and E. Purified AChR was analyzed for the presence of the protein tyrosine kinases by immunoprecipitating with anti-*fyn* or anti-*fyk* antibodies followed by incubation of the precipitates under phosphorylating conditions. Phosphorylated bands of 50, 58, and 65 kDa are observed in the antiprotein tyrosine kinase but not preimmune serum precipitates (FIG. 7D). Analysis of the precipitates by Western blotting demonstrates that the phosphorylated bands comigrate with the β, γ, and δ AChR subunits (FIG. 7D and E). These data indicate that Fyn and Fyk, in a complex with the AChR, copurify with the receptor on acetylcholine-affinity resin, that the receptor is subsequently coimmunoprecipitated with the kinases, and that both Fyn and Fyk are capable of phosphorylating the AChR in the precipitate.

Molecular Basis for the Association of the AChR with Fyn and Fyk

Like other members of the Src class of protein tyrosine kinases,[34] Fyn and Fyk each contain a unique region, an SH3 domain, an SH2 domain, and a catalytic domain. SH2 domains mediate the association of Src-like tyrosine kinases as well as a variety of other SH2 domain containing signaling components with phosphotyrosine containing proteins, including autophosphorylated growth factor receptors and tyrosine kinase substrates.[27] Therefore, we investigated the involvement of the SH2 domains of Fyn and Fyk in the association of the AChR with these two protein tyrosine kinases.

Using glutathione-S transferase fusion proteins as affinity reagents, we examined whether the coimmunoprecipitation of the AChR with Fyn and Fyk is mediated by binding of the receptor to the SH2 domains. Upon incubation of solubilized *Torpedo* electric organ postsynaptic membrane proteins representing between 30 and 1 μg of protein with the affinity reagents, the AChR binds to the Fyn and Fyk SH2 domain fusion proteins in a concentration-dependent manner (FIG. 8). By comparing the relative intensity of the AChR detected in the flow through to that in the bound, we estimate that between 5% (30 μg) and 20% (1 μg) of the receptor binds to the Fyn and Fyk SH2 affinity resin under these conditions. The binding of the AChR to the SH2 domain fusion proteins is specific because little if any AChR binds to the glutathione-S transferase backbone upon incubation with the highest concentration of protein (30 μg).

The binding of the AChR to the Fyn and Fyk SH2 domain fusion proteins could be mediated by an ancillary protein or may be due to a direct interaction of one or more receptor subunits with the kinase fragment. To address this question, postsynaptic membrane proteins were treated under denaturing conditions, 2% SDS, to dissociate the AChR from any bound proteins as well as to dissociate the receptor into individual subunits. After dilution of the membrane proteins into Triton X-100, we examined the ability of the individual receptor subunits to bind to the SH2 fusion proteins. After solubilization under native conditions, incubation of postsynaptic membrane proteins with the SH2 domain affinity resins results in AChR binding, and all four subunits are detected (FIG. 9) as demonstrated above. However, after denaturation only the AChR δ subunit is observed to bind to the Fyn and Fyk SH2 domain fusion proteins (FIG. 9). These data support a direct interaction between the AChR δ subunit and the Fyn and Fyk SH2 domain fusion proteins. In addition, these data indicate that not all tyrosine phosphorylated postsynaptic membrane proteins, including the AChR β and γ subunits, bind to Fyn and Fyk SH2 domain fusion proteins.

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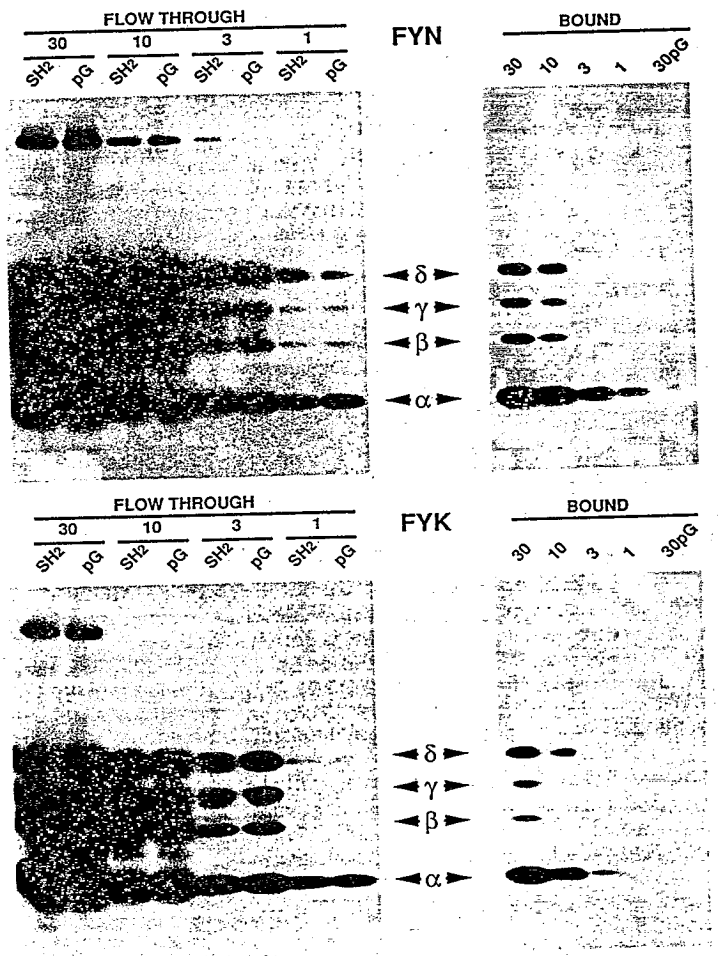

FIGURE 8. AChR binding to SH2 domain fusion protein of Fyn and Fyk. *Torpedo* electric organ postsynaptic membranes, representing between 1 and 30 μg of membrane protein, were incubated under phosphorylating conditions, solubilized, and incubated with glutathione agarose containing 5 μg of either Fyn or Fyk SH2 domain fusion protein (SH2) or glutathione-S-transferase backbone protein (pG) as described.[12] The proteins in both the supernatants (Flow Through) and those bound to the agarose (Bound) were resolved by SDS-PAGE, blotted onto nitrocellulose, and analyzed with antiserum to the AChR as described.[12] The positions of the α, β, γ, and δ subunits are as indicated. (From Swope and Huganir.[12] Reproduced, with permission, from the *Journal of Biological Chemistry.*)

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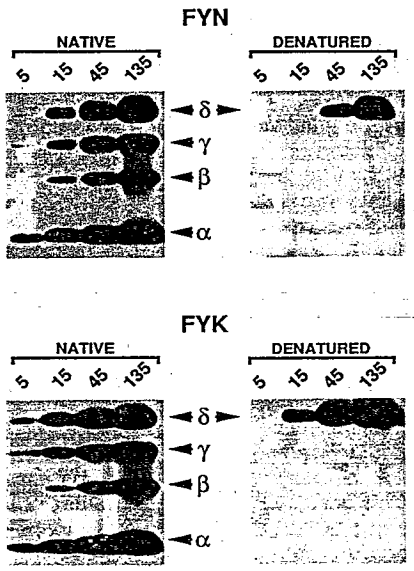

FIGURE 9. Subunit specificity for binding of the AChR to Fyn and Fyk SH2 domain fusion proteins. *Torpedo* electric organ postsynaptic membranes (100 μg) were incubated under phosphorylating conditions, treated without (Native) or with (Denatured) 2% SDS, and diluted to a final concentration of 0.4% SDS/2% Triton X-100. The solubilized proteins were incubated with glutathione agarose containing the indicated micrograms of Fyn or Fyk SH2 domain fusion protein as described.[12] The bound proteins were resolved by SDS-PAGE, blotted onto nitrocellulose, and analyzed with antiserum to the AChR as described.[12] The positions of the α, β, γ, and δ subunits are as indicated. (From Swope and Huganir.[12] Reproduced, with permission, from the *Journal of Biological Chemistry.*)

To examine whether the association of the AChR with Fyn and Fyk during immunoprecipitiation is mediated by binding of the receptor to the SH2 domain of the kinases, the effect of postsynaptic membrane protein denaturation was determined. Under native conditions, the AChR is enriched in the anti-Fyn and anti-Fyk immunoprecipitates (FIG. 10) as shown above (FIG. 7). All four AChR subunits are detected in the anti-Fyk (FIG. 10) and anti-Fyn immunoprecipitates (longer expo-

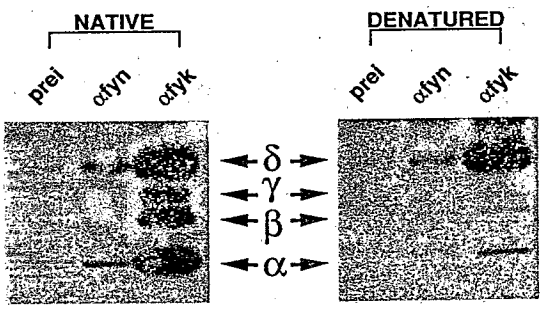

FIGURE 10. Subunit specificity for coimmunoprecipitation of the AChR with Fyn and Fyk. *Torpedo* electric organ postsynaptic membranes (250 μg) were incubated under phosphorylating conditions, treated without (Native) or with (Denatured) 2% SDS, and diluted to a final concentration of 0.4% SDS/2% Trition X-100. The solubilized proteins were immunoprecipitated with preimmune (prei), anti-*fyn* (αfyn), or anti-*fyk* (αfyk) serum, resolved by SDS-PAGE, and analyzed by Western blot using a pool of monoclonal antibodies to the AChR as described.[12] The positions of the α, β, γ, and δ subunits are as indicated. (From Swope and Huganir.[12] Reproduced, with permission, from the *Journal of Biological Chemistry.*)

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sures of film; data not shown). Upon denaturation, the anti-Fyk immunoprecipitate is specifically enriched in the AChR δ subunit (FIG. 10). In addition, as shown in FIGURE 10 and by scanning densitometry (data not shown), after denaturation the anti-Fyn immunoprecipitate is also enriched in the AChR δ subunit as well as the β subunit. These data indicate that coimmunoprecipitation of the AChR with Fyn and Fyk is mediated by a direct association of the receptor with the protein tyrosine kinases. In addition, the fact that the dissociated AChR δ subunit coimmunoprecipitates with Fyn and Fyk is consistent with the results obtained using the fusion proteins and supports an association of the receptor with the kinases via the SH2 domains of Fyn and Fyk.

DISCUSSION

The results presented here demonstrate that agrin induces the clustering of AChR and phosphotyrosine-containing proteins. One of these phosphoproteins appears to be the AChR itself, because agrin induces the tyrosine phosphorylation of the receptor. Whether agrin-induced AChR phosphorylation mediates receptor clustering or is a consequence of clustering is not presently known. One attractive hypothesis is that agrin-induced tyrosine phosphorylation of the AChR β subunit of the AChR might alter its interaction with the cytoskeletal elements in such a way as to cause receptor immobilization. At developing neuromuscular junctions, phosphorylated receptors would accumulate at synaptic sites as a result of the local activation of a protein tyrosine kinase by agrin released from the axon terminal. However, it is possible that agrin-induced phosphorylation of the AChR is a consequence of receptor aggregation. For example, protein tyrosine kinases might accumulate together with the AChR in agrin-induced specializations, phosphorylating receptors as they aggregate.

We used the electric organ of *Torpedo californica* as a system for identifying protein tyrosine kinases involved in the regulation of synaptic function. Two kinases of the Src class were identified, cloned, sequenced, and functionally characterized. As demonstrated by examining the homology of the conserved and unique regions of our two clones to known protein tyrosine kinases, one of the clones is the *Torpedo* homolog of neuronal Fyn. The other is a novel protein tyrosine kinase we have named Fyk because of its homology to the Fyn and Yes protein tyrosine kinases.

As for many other types of protein tyrosine kinases,[25,35,36] the activities of Fyn and Fyk are highest in the brain, suggesting an involvement in neuronal function. In neurons, protein tyrosine kinases are believed to be involved in differentiation[5,6] as well as synaptic function.[4] Our results suggest that Fyn and Fyk are important for postsynaptic function at the neuromuscular junction and may be involved in the tyrosine phosphorylation of the AChR. For example, the enrichment of Fyn and Fyk activities in the electric organ as compared to muscle suggests that the two kinases are not simply "housekeeping" proteins of muscle and electroplax, which is derived from muscle, but are coenriched in electric organ with other synaptic proteins. In addition, both kinases are detected in the postsynaptic membrane fraction that is enriched in the AChR; Fyn and Fyk comprise 36% and 8%, respectively, of the total protein tyrosine kinase activity. Furthermore, our data demonstrate that anti-AChR immunoprecipitates contain a protein tyrosine kinase activity that phosphorylates the receptor. Conversely, the AChR but not the 43-kDa protein is enriched in immunoprecipitates of both Fyn and Fyk. Finally, phosphorylation of the AChR β, γ, and δ subunits occurs when affinity-purified AChR is immunoprecipitated with anti-*fyn* and anti-*fyk* antibodies followed by incubation under phosphorylating condi-

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tions. These data indicate that Fyn and Fyk form a tight complex with the AChR and suggest that the coimmunoprecipitated AChR is phosphorylated by these protein tyrosine kinases. In addition, these data suggest that Fyn and Fyk may catalyze the endogenous tyrosine phosphorylation of the AChR.

The N-terminal half of Src-like protein tyrosine kinases contains four discrete functional domains including the SH2 domain.[34] The SH2 domain was originally identified as a noncatalytic region that is conserved among cytoplasmic protein tyrosine kinases and modifies kinase function.[37] Subsequently, SH2 domains were shown to be contained in a variety of signaling proteins, playing a crucial role in their association with activated growth factor receptors.[27,38] SH2 domains bind tyrosine phosphorylated proteins including autophosphorylated protein tyrosine kinases and tyrosine kinase substrates[27] via the recognition of specific phosphopeptide sequences.[39,40] Inasmuch as the AChR is a substrate for a protein tyrosine kinase(s), we postulated that the coimmunoprecipitation of the receptor with Fyn and Fyk may be mediated by an association between the SH2 domain of each kinase with the tyrosine-phosphorylated receptor. To address this possibility, we employed affinity chromatography using fusion proteins derived from the SH2 domain of Fyn and Fyk.

We found that the AChR bound to the SH2 domain fusion proteins in a manner dependent on protein concentration. As demonstrated by two independent methods, the binding of the AChR to the SH2 domains requires tyrosine phosphorylation. First, the effect of several phosphoamino acids on AChR binding was determined; both phosphotyrosine and phenylphosphate block binding whereas phosphoserine and phosphothreonine do not.[12] In addition, the extent of AChR binding to the Fyn and Fyk affinity resin correlates with the extent of receptor tyrosine phosphorylation.[12]

The AChR is a 250-kDa pentameric complex comprising four homologous transmembrane subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$.[7] The β, γ, and δ subunits are each phosphorylated on a single tyrosine residue that is contained on the major intracellular loop between transmembrane domains three and four.[41] We investigated the subunit specificity for the binding of the AChR to the Fyn and Fyk SH2 domain fusion proteins. Subsequent to dissociation of the AChR by SDS, the binding of the individual receptor subunits to the affinity resin was examined. A dramatically specific binding of the δ subunit to the Fyn and Fyk SH2 fusion protein was observed. These data suggest that only the δ subunit binds directly to the SH2 domain affinity resin. Alternatively, the binding sites on the β and γ subunits may have been inactivated by the SDS treatment. Therefore, the subunit specificity for binding of the intact native AChR was examined using phosphotyrosine-containing peptides derived from the known phosphorylation sites of the receptor.[41] The δ subunit phosphopeptide is at least 10-fold more potent than the β or γ subunit phosphopeptides in blocking the binding of the native AChR to the Fyn and Fyk SH2 domain fusion proteins.[12] Examination of the peptide sequence for the β, γ, and δ subunits reveals that the subunit specificity observed here is in agreement with previous reports. Phosphopeptides containing asparagine at position +2 to the phosphotyrosine and especially isoleucine at postion +3 are preferred for binding by Src and Fyn SH2 domains.[40] The sequences of the tyrosine phosphorylation sites of the β, γ, and δ subunits are consistent with these data; the δ subunit sequence is YFNI, whereas the β and γ subunit sequences are YFIR and YILK, respectively.[41] Thus, the association of the AChR with the SH2 domain of Fyn and Fyk appears to be mediated by the tyrosine phosphorylation site contained within the major intracellular loop between transmembrane domains three and four of the receptor δ subunit.

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The AChR specifically coimmunoprecipitates with Fyn and Fyk. To examine whether the association of the AChR with Fyn and Fyk is mediated by binding of the receptor to the SH2 domains of the kinases, the effect of postsynaptic membrane protein denaturation was determined. In agreement with the results using SH2 domain fusion protein affinity resin, the coimmunoprecipitation of the AChR with Fyn and Fyk appears to be mediated by the δ subunit: after denaturation by SDS, the anti-*fyn* and anti-*fyk* immunoprecipitates are also enriched in the AChR δ subunit. Thus, binding of the tyrosine-phosphorylated intracellular loop of the AChR δ subunit with the SH2 domain of Fyn and Fyk appears to mediate the association of the receptor with these two protein tyrosine kinases.

The functional consequence of association between the AChR and Fyn and Fyk remains to be determined. It has been postulated that binding of tyrosine-phosphorylated proteins to the SH2 domain of Src-like kinases and adapter proteins stabilizes the bound substrates by preventing rapid dephosphorylation.[27] In fact, binding of Src SH2 domain fusion protein to pp^{125} focal adhesion kinase, protects the major site of pp^{125} focal adhesion kinase autophosphorylation from dephosphorylation upon treatment with a tyrosine phosphatase *in vitro*.[42] Alternatively, association between tyrosine-phosphorylated substrates and SH2 domain containing proteins may provide a means for forming complexes of proteins containing a variety of functions. Thus, association between Fyn and Fyk and the AChR may protect the receptor from dephosphorylation. However, the association may promote the recruitment of enzymatic and structural elements involved in synaptic transmission at the neuromuscular junction.

Synaptogenesis is a complex developmental process for which the neuromuscular junction has served as a model. The clustering of postsynaptic components at the nerve-muscle contact appears to be regulated by factors contained in the extracellular matrix[43] including agrin[44,45] and basic fibroblast growth factor.[46,47] The action of these components may be mediated by tyrosine phosphorylation. In fact, a role for protein tyrosine kinase activation has been implicated in the clustering of synaptic components induced by a variety of neuronal and nonneuronal stimuli including nerve,[9] polymer beads,[48,49] electric field,[50] expression of exogenous 43-kDa protein,[51] and *in vitro* treatment with basic fibroblast growth factor[47] or agrin.[10] Formation of the postsynaptic specializations is associated with a rearrangement of cytoskeletal elements[52] and an alteration in gene expression.[53] The ubiquitous expression of Src-like protein tyrosine kinases as well as their substrate promiscuity suggests that these kinases regulate a wide range of cellular functions.[29,54] Although the data presented here indicate that Fyn and Fyk function to phosphorylate the AChR, it will be interesting to examine additional roles of these two protein tyrosine kinases during synaptogenesis.

The molecular mechanisms involved in the regulation of Fyn and Fyk in the postsynaptic membrane of *Torpedo* electric organ and in skeletal muscle are not known. Tyrosine phosphorylation of the AChR is regulated by innervation in rat skeletal muscle,[9] a process that appears to be mediated by agrin.[10,51] Agrin interacts with a specific receptor in the muscle plasma membrane that is responsible for the nerve and agrin-induced clustering of the AChR at the synapse.[55,56] An agrin receptor has recently been identified as the extracellular peripheral membrane subunit of α-dystroglycan.[57–59] It will be interesting to determine whether this agrin binding protein functions to transduce the activation of an intracellular protein tyrosine kinase such as Fyn and/or Fyk.

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SUMMARY

Most neurotransmitter receptors examined to date are either regulated by phosphorylation or contain consensus sequences for phosphorylation by protein kinases. The nicotinic acetylcholine receptor (AChR), which mediates depolarization at the neuromuscular junction, has served as a model for the study of the structure, function, and regulation of ligand-gated ion channels. The AChR is phosphorylated by protein kinase A, protein kinase C, and an unidentified protein tyrosine kinase. Tyrosine phosphorylation of the AChR is correlated with a modulation of the rate of receptor desensitization and is associated with AChR clustering. We showed that agrin, a neuronally derived extracellular matrix protein, induces AChR clustering and tyrosine phosphorylation. In addition, we identified two protein tyrosine kinases, Fyn and Fyk, that appear to be involved in the regulation of synaptic transmission at the neuromuscular junction by phosphorylating the AChR. The two kinases are highly expressed in *Torpedo* electric organ, a tissue enriched in synaptic components including the AChR. As demonstrated by coimmunoprecipitation, Fyn and Fyk associate with the AChR. Furthermore, the AChR is phosphorylated in Fyn and Fyk immunoprecipitates. We investigated the molecular basis for the association of the AChR with Fyn and Fyk using fusion proteins derived from the kinases. The AChR bound specifically to the SH2 domain fusion proteins of Fyn and Fyk. The association of the AChR with the SH2 domains is dependent on the state of AChR tyrosine phosphorylation and is mediated by the δ subunit of the receptor. These data provide evidence that the protein tyrosine kinases Fyn and Fyk may act to phosphorylate the AChR *in vivo*.

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